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Rhizophagus natalensis, a new species in the *Glomeromycota*

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Abstract — Morphological and histochemical studies of spores and phylogenetic analyses of LSU and SSU nrDNA sequences have indicated that a fungal species found in maritime dunes of the Parque Estadual das Dunas de Natal "Journalista Luiz Maria Alves" located in Natal, Brazil, represents an undescribed species of *Rhizophagus*. The new species, *R. natalensis*, forms spores singly or (sometimes) in loose clusters in soil. Its spores are pastel yellow to light yellow and globose to subglobose [(75–)101(–133) µm diam] or (rarely) ovoid (50–70 × 63–79 µm) and have one subtending hypha with a pore that is open or occluded by a septum. Its spore wall comprises four layers: (1) a semi-permanent hyaline layer forming the spore surface, (2) a permanent hyaline unit layer, (3) a laminate pastel yellow to light yellow layer, and (4) a flexible hyaline layer. Layers 1 and 3 stain in Melzer's reagent.

Key words — arbuscular mycorrhizal fungi, molecular phylogeny, tropical ecosystem

Introduction

Arbuscular mycorrhizal fungi (AMF) of the phylum *Glomeromycota* live in symbiosis with ca. 70–90% of land plants growing in different habitats (Smith & Read 2008, Brundrett 2009). Sand dunes are especially favorable sites for development of AMF, mainly due to the sparse populations of other microorganisms competing for nutrients produced by living plants and the rare occurrence of AMF microparasites (Błaszkowski 1994, Dalpé 1989, Koske 1987, Lee & Koske 1994, Tadych & Błaszkowski 2000). On the other hand, the existence of plants growing in extremely poor and harsh dune sites may well depend highly on the establishment of such symbioses, in that AMF frequently increase the supply of nutrients to plants and decrease their sensitivity to

different abiotic and biotic stresses (Bothe et al. 2010, Schönbeck 1978, Dehn & Schüepp 1989, Griffioen & Ernst 1989, Smith & Read 2008).

The approximately 250 AMF species that have been described (Schüßler & Walker 2010) probably represent less than 5% of world's AMF species (Krüger et al. 2009). Of the described species ca. 62% produce glomoid spores, i.e., spores similar in mode of formation, spore wall structure, and subtending hyphal characters to those of *Glomus macrocarpum* Tul. & C. Tul. (type species of the genus *Glomus;* Clements & Shear 1931).

Glomoid spores are produced by AMF of the genus *Rhizophagus* P.A. Dang. in the family *Glomeraceae* Piroz. & Dalpé (Redecker et al. 2013; Schüßler & Walker 2010). Long after Dangeard (1896) described *Rhizophagus,* Gerdemann & Trappe (1974) synonymized the genus with *Glomus*. Recently, however, Schüßler & Walker (2010) resurrected *Rhizophagus* based on both the original description of its type species, *R. populinus* P.A. Dang., stating that the fungus produced spores abundantly inside roots, and the phylogenetic sequence analyses of *Rhizophagus* and other AMF that tend to form intraradical spores.

We studied the morphological and histochemical characters of glomoid spores extracted from a pot trap culture inoculated with rhizosphere soil and root fragments of an unrecognized grass colonizing maritime sand dunes of the Parque Estadual das Dunas de Natal "Journalista Luiz Maria Alves" located in Natal, Brazil. Our research suggested that we had found an undescribed species similar to *R. fasciculatus* (Thaxt.) C. Walker & A. Schüssler. Later phylogenies inferred from analyses of sequences of the large (LSU) and small (SSU) subunit genes support our original hypothesis that the two fungi represent different species. The Brazilian fungus is described below as *R. natalensis*.

Materials & methods

Establishment and growth of trap and single-species cultures, extraction of spores, and staining of mycorrhiza

Spores examined in this study derived from a pot trap culture. The trap culture was established to obtain living spores and to initiate sporulation of specimens that may not have sporulated in the field collections (Stutz & Morton 1996). Methods used to establish the trap culture, growth conditions, and spore extraction follow Błaszkowski et al. (2012). The growing substrate of the trap culture was the field-collected rhizosphere soil and roots of the plant species sampled mixed with autoclaved coarse-grained sand (1:1 v/v).

Eight single-species cultures were also established and grown as given in Błaszkowski et al. (2012). The single cultures were set up with ca. 10 spores and small clusters of spores (5–12) attached by a common mycelium. Unfortunately, all cultures failed.

Microscopy and nomenclature

Morphological features of spores and their wall structure were determined after examining at least 100 spores mounted in water, lactic acid, polyvinyl alcohol/lactic

acid/glycerol (PVLG; Omar et al. 1979), and a mixture of PVLG and Melzer's reagent (1:1, v/v). Glomerospores at all developmental stages were mounted in PVLG and PVLG+Melzer's reagent, then covered with a cover slip, crushed to varying degrees by applying pressure to the cover slip, stored at 65°C for 24 h to clear their contents from oil droplets, and examined under an Olympus BX 50 compound microscope equipped with Nomarski differential interference contrast optics. Microphotographs were recorded on a Sony 3CCD color video camera coupled to the microscope.

Spore structure terminology follows Stürmer & Morton (1997) and Walker (1983). Spore color was examined under a dissecting microscope on fresh specimens immersed in water. Color names are from Kornerup & Wanscher (1983). Nomenclature of fungi and the authors of fungal names are from Index Fungorum (http://www.indexfungorum.org/AuthorsOfFungalNames.htm). Voucher specimens were mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides and deposited in the Department of Ecology and Protection of Environment (DEPE), West Pomeranian University of Technology in Szczecin, Szczecin, Poland, and in the herbarium at Universidade Federal do Rio Grande do Norte (UFRN) in Natal, Rio Grande do Norte, Brazil.

DNA extraction, polymerase chain reaction and DNA sequencing

Crude DNA was isolated from 3–8 single spores crushed with a needle in ultra clean water on sterile microscope slides under a dissecting microscope. Amplification, cloning, and sequencing procedures followed Błaszkowski et al. (2012). The large subunit nrDNA gene (partial) of the fungus newly described here was amplified using the primers LR1 (Tuinen et al. 1998) and FLR2 (Trouvelot et al. 1999) and then the primers 28G1 and 28G2 (Silva et al. 2006), and SSU nrDNA gene (partial) was amplified with the primers AML1 and AML2 (Lee et al. 2008) as described in Błaszkowski et al. (2012). The segment spanning SSU (partial), internal transcribed region (ITS1, 5.8S and ITS2, full), and LSU (partial) nrDNA of *Septoglomus africanum* (Błaszk. & Kovács) Sieverd. et al., *S. furcatum* Błaszk. et al., *S. fuscum* Błaszk. et al., and *S. xanthium* (Błaszk. et al.) G.A. Silva et al. were amplified using a nested procedure and the SSUmAf–LSUmAr primer pair for the first nested PCR and the SSUmCf–LSUmBr primer pair for the second nested PCR, as suggested by Krüger et al. (2009). Representatives of sequences have been deposited in GenBank (KJ210823–KJ210828).

Sequence alignment and phylogenetic analyses

The glomeromycotan origin of the sequences was initially tested by BLAST (Zhang et al. 2000) search. To determine the generic affiliation of our new species we performed pilot phylogenetic analyses separately with all LSU and SSU sequences and those representing all recognized glomeromycotan genera with glomoid spores available in GenBank and published by Krüger et al. (2012). The results of the SSU sequence analyses are not presented here. The final data set used to generate the LSU tree in Fig. 9 comprised six sequences from our new species, three sequences each from known *Rhizophagus* spp. and one to three sequences, published or obtained by us, from 12 other species in the *Glomeraceae*. Sequences representing *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüssler served as outgroup in all analyses. The LSU and SSU sequences were aligned with PRANK$_{F+}$ with the $_{F+}$ option invoked

to fix already inferred indels at their place and avoid another indel being inferred in an overlapping position during the second recursion of the algorithm (Löytynoja & Goldman 2008). Overhanging sequence fragments of both termini were trimmed. Bayesian (BI) analyses were performed with MrBayes 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003), and maximum likelihood (ML) analyses with PHYML (Guindon & Gascuel 2003). Before the analyses the best-fit substitution models for the alignments were estimated by the Akaike information criterion (AIC) using TOPALi v. 2.5 (Milne et al. 2004). In the BI analyses of both LSU and SSU sequences the model employed was GTR + G, and TrN + G was applied in the ML analyses of both types of sequences. In the BI analyses the Markov chain was run for 5,000,000 generations, sampling in every 500 steps with a burn-in at 3000. In the ML analyses the transition/transversion ratio for DNA models and the gamma distribution parameter were estimated. Six substitution rate categories were set. Topology and branch lengths and rate parameters were optimized. Support of branches in the ML analyses was estimated in bootstrap analyses with 1000 replicates. The details of the analyses are available on request. Phylogenetic trees were visualized and edited in MEGA5 (Tamura et al. 2011).

Taxonomy

Rhizophagus natalensis Błaszk., Chwat & B.T. Goto, **sp. nov.** Figs 1–8

MycoBank MB 808282

Differs from *Rhizophagus irregularis* in spore color and shape and the number and phenotypic and histochemical characters of the spore wall layers.

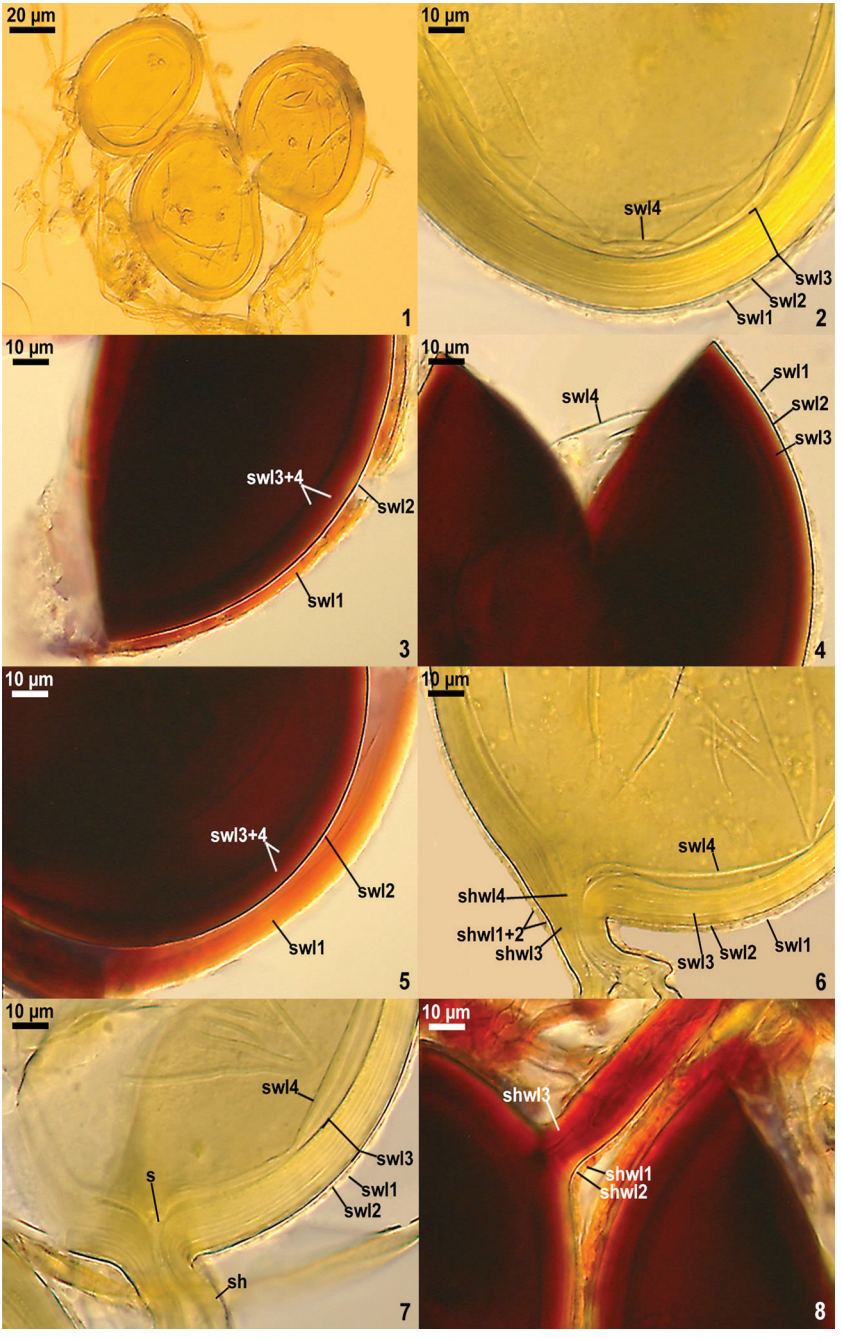
Type: Brazil, Rio Grande do Norte, Natal, Parque Estadual das Dunas de Natal "Journalista Luiz Maria Alves", under an unrecognized grass colonizing maritime sand dunes, 12 Sept. 2012, J. Błaszkowski 3381 (**Holotype**, DEPE; GenBank KJ210823), J. Błaszkowski 3382–3384 (**Isotypes**, DEPE; GenBank KJ210824–KJ210828).

Etymology. Latin, *natalensis*, referring to the 'Natal' dunes from which the species was collected.

Sporocarps unknown.

Glomerospores formed singly, sometimes in loose clusters, in soil; developing blastically at the tip of sporogenous hyphae either directly from mycorrhizal extraradical hyphae (single spores) or branched from a parent hypha continuous with a mycorrhizal extraradical hypha (spores in clusters). Clusters 160–989 × 180–1200 µm with 2–14 spores. Spores pastel yellow (3A4) to light yellow (3A5); globose to subglobose; (75–)101(–133) µm diam; rarely ovoid; 50–70 × 63–79 µm; with one subtending hypha.

Figs. 1–8. *Rhizophagus natalensis* spores (differential interference microscopy). **1**. Spores in loose cluster. **2–5**. Spore wall layers (swl) 1–4; note the unstained layer 4 in Fig. 4 and the swollen layer 1 in Fig. 5. **6**. Spore wall layers (swl) 1–4 continuous with subtending hyphal wall layers (shwl) 1–4; note shwl4 extending along the inner surface of shwl3. **7**. Spore wall layers (swl) 1–4, of which layer 4 forms a curved septum (s) in the subtending hyphal (sh) lumen. **8**. Subtending hyphal wall layers (shwl) 1–3 continuous with spore wall layers 1–3; shwl1 and swl1 are not visible. Figs 1, 2, 6, 7, in PVLG; Figs 3–5, 8, in PVLG+Melzer's reagent.


20 µm
1
10 µm
swl4
swl3
swl2
swl1
2
10 µm
swl3+4
swl2
swl1
3
10 µm
swl4
swl1
swl2
swl3
4
10 µm
swl3+4
swl2
swl1
5
10 µm
swl4
shwl4
shwl1+2
shwl3
swl3
swl2
swl1
6
10 µm
swl4
swl3
s
swl1
swl2
sh
7
10 µm
shwl3
shwl1
shwl2
8

Spore wall consists of four layers: layer 1, forming the spore surface, semi-permanent, hyaline, (1.0–)2.4(–5.3) µm thick, slowly deteriorating with age, usually present as a more or less decomposed structure even in older spores; sometimes swelling and becoming up to 8.3 µm thick; layer 2, a unit layer, permanent, smooth, hyaline, (0.8–)1.1(–1.5) µm thick; layer 3 laminate, smooth, pastel yellow (3A4) to light yellow (3A5), (6.3–)8.7(–14.0) µm thick, consisting of laminae up to 0.8–1.0 µm thick, frequently easily separating from each other in crushed spores; and layer 4 flexible, smooth, hyaline, (0.8–)1.3(–2.0) µm thick, usually separating from the lower surface of layer 3 in crushed spores. Layers 1 and 3 stain reddish white (9A2) to greyish red (10C5) and brownish violet (11D8) to violet brown (11E8) in Melzer's reagent, respectively.

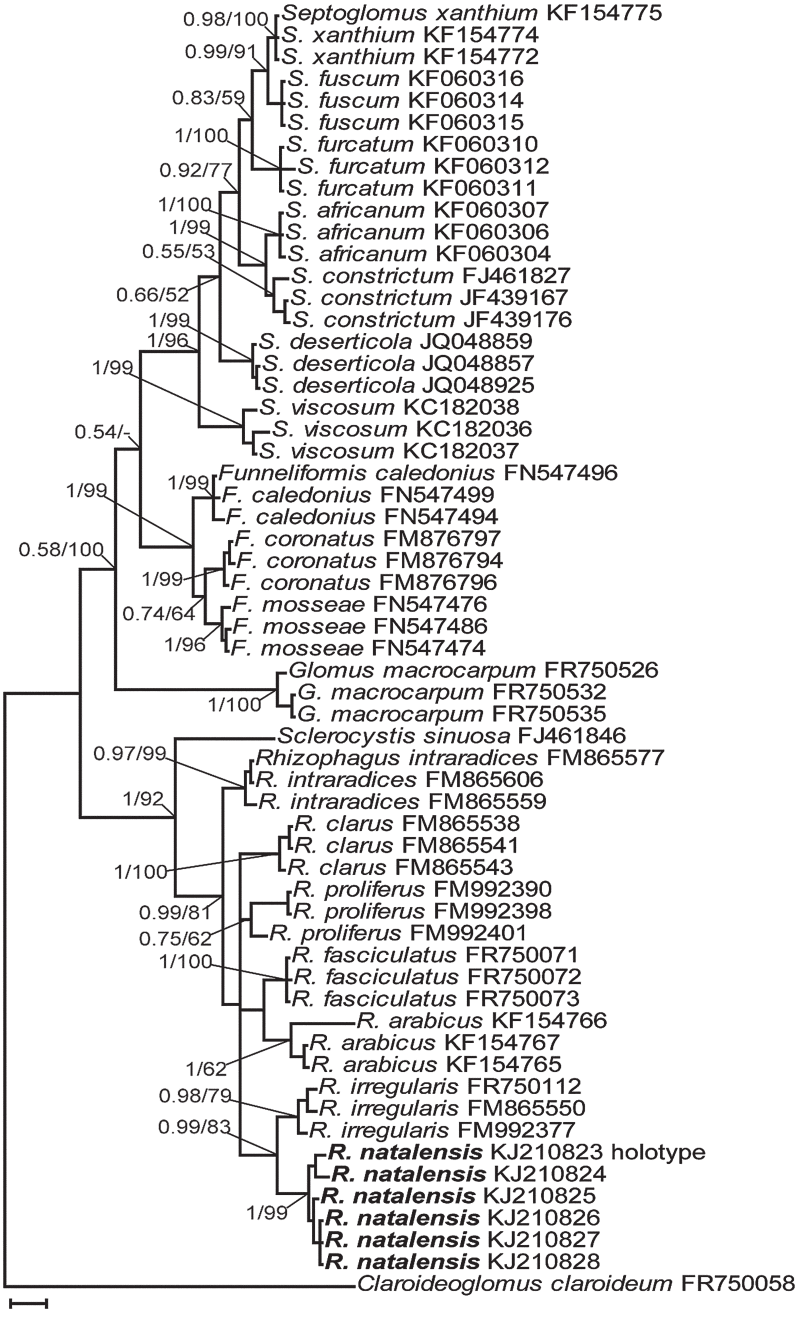
Subtending hypha pastel yellow (3A4) to light yellow (3A5); straight or recurved, cylindrical to funnel-shaped, sometimes slightly constricted at the spore base; (16.0–)20.8(–29.8) µm wide at the spore base. Wall of subtending hypha pale pastel yellow (3A4) to light yellow (3A5); (7.3–)9.4(–14.3) µm thick at the spore base; continuous with spore wall layers 1–4. Pore (1.5–)2.4(–3.3) µm diam, open when spore wall layer 4 develops from subtending hyphal wall layer 4 arising far below the spore base and extending along the inner surface of subtending hyphal wall layer 3 or occluded by a curved septum continuous with spore wall layer 4 and open or occluded from the reasons mentioned above and gradually narrowing due to thickening of wall layer 3 of the subtending hypha towards the centre of its lumen.

Germination unknown.

Mycorrhizal associations — In the field *R. natalensis* was associated with roots of an unrecognized plant species of *Poaceae* that colonized the Atlantic Ocean sand dunes in the Parque Estadual das Dunas de Natal "Journalista Luiz Maria Alves" (5°46′S, 35°12′W), one of the largest urban conservation areas with dune vegetation in Brazil. In a trap culture inoculated with the rhizosphere soil and root fragments of the plant, the fungus lived in symbiosis with *Plantago lanceolata* and sporulated abundantly. However, *R. natalensis* stopped producing spores after ca. two years of growing of this culture. All attempts to establish single-species cultures from spores extracted from the trap culture failed.

Phylogenetic position — In BI and NJ trees with LSU sequences the *R. natalensis* clade obtained high values of statistical support and was sister to

Fig. 9. 50% majority rule consensus phylogram of AMF species inferred from a Bayesian analysis of LSU nrDNA sequences of our new species and 33 other species, including *Claroideoglomus claroideum* as outgroup. The new species is shown in boldface. The fungal names are followed by GenBank accession numbers. The Bayesian posterior probabilities ≥0.50 and ML and NJ bootstrap values ≥50% are shown near the branches, respectively. Bar indicates 0.5 expected change per site per branch.


0.98/100
Septoglomus xanthium KF154775
S. xanthium KF154774
0.99/91
S. xanthium KF154772
S. fuscum KF060316
0.83/59
S. fuscum KF060314
S. fuscum KF060315
1/100
S. furcatum KF060310
0.92/77
S. furcatum KF060312
S. furcatum KF060311
1/100
S. africanum KF060307
1/99
S. africanum KF060306
0.55/53
S. africanum KF060304
S. constrictum FJ461827
0.66/52
S. constrictum JF439167
1/99
S. constrictum JF439176
1/96
S. deserticola JQ048859
1/99
S. deserticola JQ048857
S. deserticola JQ048925
S. viscosum KC182038
0.54/-
S. viscosum KC182036
S. viscosum KC182037
Funneliformis caledonius FN547496
1/99
1/99
F. caledonius FN547499
F. caledonius FN547494
F. coronatus FM876797
0.58/100
F. coronatus FM876794
1/99
F. coronatus FM876796
F. mosseae FN547476
0.74/64
F. mosseae FN547486
1/96
F. mosseae FN547474
Glomus macrocarpum FR750526
1/100
G. macrocarpum FR750532
G. macrocarpum FR750535
Sclerocystis sinuosa FJ461846
0.97/99
Rhizophagus intraradices FM865577
R. intraradices FM865606
1/92
R. intraradices FM865559
R. clarus FM865538
R. clarus FM865541
1/100
R. clarus FM865543
R. proliferus FM992390
0.99/81
R. proliferus FM992398
0.75/62
R. proliferus FM992401
1/100
R. fasciculatus FR750071
R. fasciculatus FR750072
R. fasciculatus FR750073
R. arabicus KF154766
R. arabicus KF154767
1/62
R. arabicus KF154765
R. irregularis FR750112
0.98/79
R. irregularis FM865550
0.99/83
R. irregularis FM992377
R. natalensis KJ210823 holotype
R. natalensis KJ210824
R. natalensis KJ210825
1/99
R. natalensis KJ210826
R. natalensis KJ210827
R. natalensis KJ210828
Claroideoglomus claroideum FR750058

a clade with *R. irregularis* (Błaszk. et al.) C. Walker & A. Schüssler (Fig. 9). In BI and ML trees with SSU sequences *R. natalensis* was strongly supported as monophyletic and sister to a clade comprising *R. fasciculatus*, *R. intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüssler, *R. irregularis*, and *R. vesiculiferus* (Thaxt.) C. Walker & A. Schüssler distributed into two subclades (data not shown).

Distribution & habitat — Spores of *R. natalensis* were found only in one pot trap culture inoculated with a mixture of the rhizosphere soil and root fragments of an unrecognized plant species of *Poaceae* growing in maritime sand dunes of the Parque Estadual das Dunas de Natal "Journalista Luiz Maria Alves" located in Natal, Brazil. Data on the climate, vegetation and soil chemical properties of the dunes in which *R. natalensis* occurred were described previously (Goto et al. 2012).

Spores of *R. natalensis* were not found in either ca. 3000 field-collected soils and ca. 3000 pot trap cultures representing different regions of Europe, Africa, Asia, Cuba, and the U.S.A. (Błaszkowski, pers. observ.).

However, BLAST queries indicated that the *R. natalensis* LSU sequences we obtained were 99% similar to: (1) five LSU sequences (FR871319, FR871318, FR871325, FR871323, FR871320) of AMF colonizing roots and rhizosphere soils of *Brachypodium retusum* (Pers.) P. Beauv. and *Bromus rubens* L. growing in semiarid degraded area located in the natural ecological park "Vicente Blanes" in Molina de Segura, Province of Murcia, southern Spain (Torrecillas et al. 2012); (2) an LSU sequence (EF066656) of an AMF coming from soil collected under *Medicago* spp. cultivated in a Mediterranean fallow land located at the Mas d'Imbert, France (Pivato et al. 2007); and (3) 55 SSU sequences of uncultured AMF from, e.g., Spain (Alguacil et al. 2012; Torrecillas et al. 2012). This suggests that *R. natalensis* has a wide world distribution.

Discussion

The distinguishing morphological and histochemical characters of *R. natalensis* are its light yellow, relatively large spores with a four-layered spore wall, of which layers 1 and 3 stain intensively in Melzer's reagent. In addition, based on DNA sequence analyses, the species is phylogenetically unique.

Our LSU sequence analyses indicate that *R. natalensis* is most closely related to *R. irregularis* (see "Phylogenetic position" above and Fig. 9). Mature *R. irregularis* spores are frequently hyaline (vs. no mature hyaline spores in *R. natalensis*) and ovoid to oblong or irregular with deep local depressions (Błaszkowski 2012; Błaszkowski et al. 2008; vs. usually globose to subglobose without depressions). The spore wall of *R. irregularis* comprises three (vs. four) layers, of which layer 1 frequently is highly thickened at the spore top and forming a cap-like swell (vs. no such thickening), a laminate innermost layer

(vs. a flexible innermost layer 4), and the only spore wall component (vs. layers 1 and 3) that stains in Melzer's reagent. The subtending hypha of *R. irregularis* spores is 0.6–2.5-fold narrower and has a 1.7–4.9-fold thinner wall at the spore base and its pore may be open or closed by a septum, but the septum forms some innermost laminae of the laminate spore wall layer 3 (vs. spore wall layer 4 in *R. natalensis*). In addition, *R. irregularis* tends to form spores in oblong clusters inside roots and in soil, because the spores arise either terminally from or intercalary inside sporogenous hyphae developing from spore wall layer 1 of an earlier fully differentiated parent spore or/and a branch of the parent hypha continuous with a mycorrhizal extraradical hypha (Błaszkowski 2012; Błaszkowski et al. 2008; vs. no such mode of spore formation in *R. natalensis*).

SSU sequence analyses also support a close relationship of *R. natalensis* with *R. fasciculatus*, *R. intraradices*, and *R. vesiculiferus* (data not shown). Intact *R. natalensis* spores observed under low and high microscope magnifications are almost morphologically and histochemically identical to those of *R. fasciculatus*. Both species form spores singly and in clusters and the spores are similar in color and size and stain intensively in Melzer's reagent (Błaszkowski 2012; Morton, http://invam.wvu.edu/). Examination of spores crushed in PVLG and PVLG+Melzer's reagent readily separates both species. The spore wall of *R. fasciculatus* is 3-layered (lacking layer 2 of the 4-layered wall of *R. natalensis*). Spore wall layer 1 of *R. natalensis*, forming the spore surface, is semi-permanent (vs. permanent in *R. fasciculatus*), may be 2.0–2.9-fold thicker, sometimes swells up to 8.3 µm thick, thereby showing its plasticity (vs. no such behaviour), and may stain more intensively in Melzer's reagent (vs. reddish white; Błaszkowski 2012). In addition, the laminae of the laminate structural spore wall layer 3 of *R. natalensis* are ca. 2-fold thicker than those of the laminate structural spore wall layer 2 of *R. fasciculatus* (<0.5 µm thick) and the *R. natalensis* laminae frequently separate from each other in crushed spores (vs. usually remaining adherent in *R. fasciculatus*). Finally, the subtending hypha of *R. natalensis* spores is less regular in shape (cylindrical to funnel-shaped vs. cylindrical in *R. fasciculatus*), has a 5.2–6.5-fold thicker wall at the spore base, and its pore may be open or occluded by a septum continuous with spore wall layer 4 (vs. closed by a septum continuous with spore wall layer 3).

Freshly mature spores of *R. intraradices* usually are light yellow with a greenish tint (Błaszkowski 2012, Morton http://invam.wvu.edu/, Stürmer & Morton 1997) lacking in *R. natalensis* spores. The spore wall of *R. intraradices* is 1.3–1.8-fold thinner, comprises only three (vs. four) layers of which only the laminate layer 3 (vs. layers 2–4) is permanent, and only spore wall layer 1 (vs. layers 1 and 3) stains in Melzer's reagent. In addition, the subtending hypha of *R. intraradices* spores is less regular in shape (cylindrical to slightly flared vs. cylindrical to funnel-shaped) and 1.2–1.6-fold narrower at the spore base; its

wall is 1.8–2.2-fold thinner at the spore base and its pore is open (vs. open or closed by a septum).

Rhizophagus vesiculiferus is most clearly separated from *R. natalensis* by the formation of sporocarps with a peridium-like layer of thin-walled vesicles (Berch & Fortin 1984, Gerdemann & Trappe 1974). In addition, *R. vesiculiferus* spores are ca. 1.6-fold smaller, their spore wall is 2-layered (vs. 4-layered) and 2.2–2.9-fold thinner, and their subtending hypha is ca. 1.7-fold narrower with an open pore (vs. open or occluded by a septum).

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